HtrA1 grant progress report

CalCyte team's co-founder, Kang Zhang found that polymorphisms in the promoter of the gene encoding HTRA1 plays a major role in genetic susceptibilty to AMD. The polymorphism (SNP rs11200638) is located 512 bp upstream of the HTRA1 transcription start site and the A risk allele is exclusively associated with a major disease haplotype which increases HTRA1 expression by approximately three fold. This human genetic discovery suggests that blocking HTRA1 activity may be an important strategy for treating AMD. Kang Zhang further found that monoclonal and polyclonal antibodies to HTRA1 can effectively inhibit pathologic angiogensis in murine models of hyperoxic induced retinal vascular disease and choroidal neovascularization, which provided the first proof of concept that blocking HTRA1 could be an effective therapeutic strategy. The central goal of this SBIR grant is to advance HTRA1 small molecule inhibitors as a possible treatment for age-related macular degeneration.

To achieve this ultimate goal, CalCyte team finished the following studies and here we report the progress and result as below:

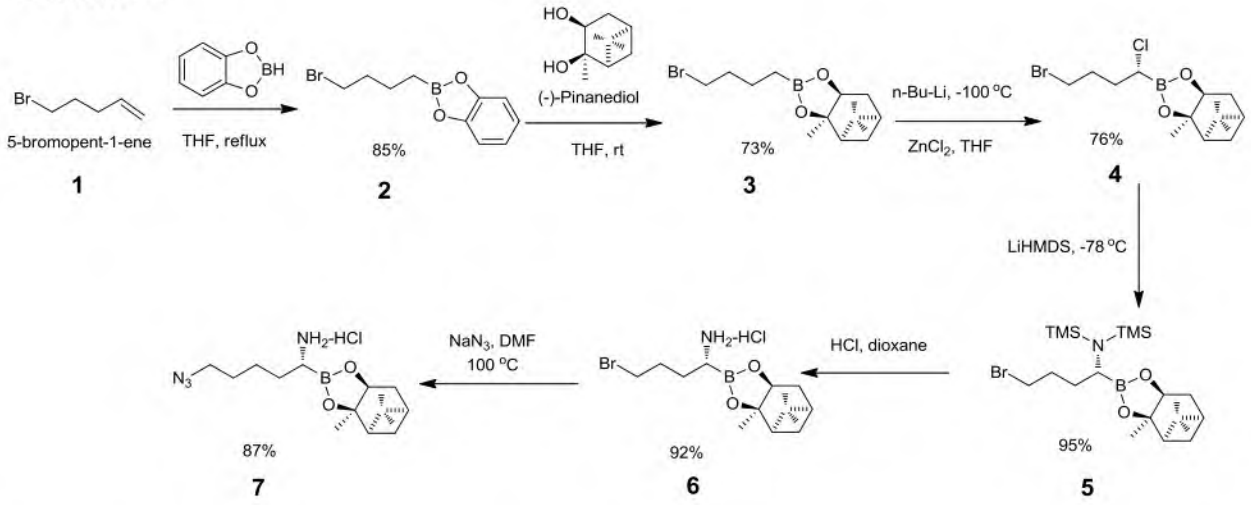
For aim 1: Validation of the efficacy of HTRA1 inhibitor NVP-LBG976 in animal models of retinal vascular disease.

Small molecule NVP-LBG976 is the key molecule we used in this study. However there is no reported synthetic protocol for it. So first we tried to establish the reliable synthesis to make 200mg of NVP-LBG976.

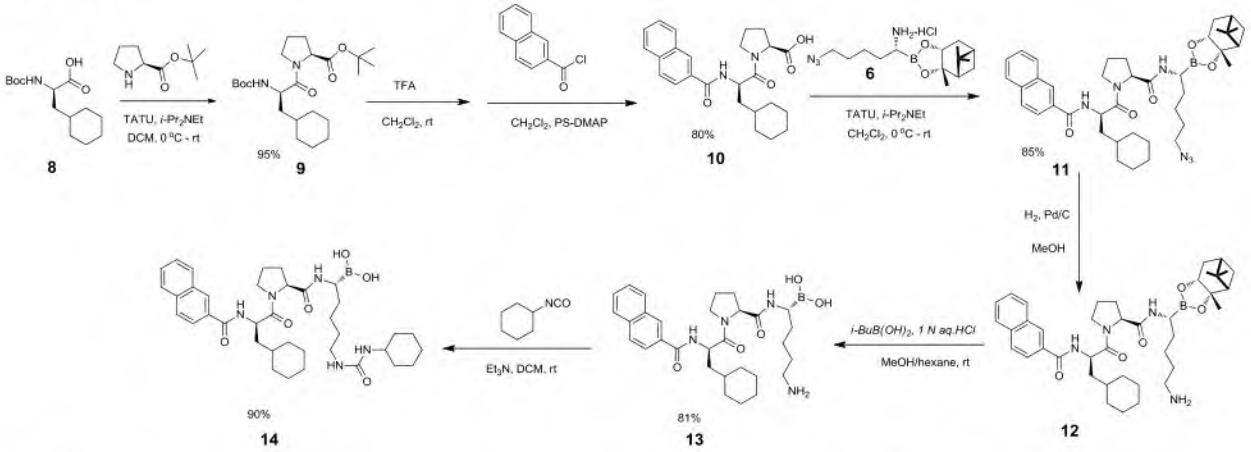
From the starting material 5-bromopent-1-ene (1) it took us 6 steps to get the key intermediate α-aminoboronate (7) with the overall yield ~35%. See scheme 1.

From the starting material amino cyclohexylpropanoic acid (8) it took us another 6 steps to get the final product NVP-LBG976 with the overall yield ~47%.

Scheme 1



Scheme 2



Then Calcyte tested NVP-LBG976 in oxygen induced retinal vascular disease (OIR, used as models of retinal neovascularization) and laser induced choroidal neovascularization (CNV, used as models of wet AMD) models.

In OIR model, Oxygen-Induced Retinopathy was induced. Seven day postpartem (P7) pups along with nursing mothers were placed in 75% oxygen, which are maintained by Pro-OX oxygen controller (BioSpherix). Pups were removed on P12 and will be given an intraocular injection of 1 µl at 5µM NVP-LBG976 or vehicle control condition. Mice were sacrificed on P17 and perfused via the left ventricle with 1ml 50mg/ml FITC-Dextran (Sigma). Eyes were enucleated, fixed for 30 minutes in 4% paraformaldehyde, and retinal flatmounts generated. Images were taken using Axiovert 200 fluorescence microscopy (Carl Zeiss). Neovascularization will be quantified using AxioVision software (Carl Zeiss). The result is as expected: eyes with intravitreal injection of 1 µl at 5µM NVP-LBG976 showed less retinal neovascularization.

In CNV model, 2-3 months adult mice were subjected to laser-induced disruption of Bruch's membrane in CNV model. A general anesthesia was introduced via intraperitoneal injection of a mixture of ketamine hydrochloride and xylazine hydrochloride. The pupil was dilated with 1% tropicamide for photocoagulation. An Iridex OcuLight GL 532 nm laser photocoagulator (Iridex) with slit lamp delivery system was used to disrupt Bruch's membrane at 3 spots at posterior pole of retina with the following parameter: 150mW power, 75um spot size, and 0.1 seconds duration. Production of a bubble at the time of laser, which indicates rupture of Bruch's membrane, was an important factor in obtaining CNV, so only burns in which a bubble produced were included in the study. Immediately after laser treatment and 3 days later, Intravitreal injection of 1 µl at 5µM NVP-LBG976 or vehicle control was performed. 7 days later the mice were sacrificed and choroidal flat mount was made after fixation. Biotin conjugated isolectin (Sigma) and Texas red conjugated streptavidin (Sigma) were used to stain blood vessel. Flat mounts were examined by Zeiss LSM 510 confocal microscope (Zeiss) and area of choroidal neovascularization was measured and quantified by ImageJ (NIH) software. Again the result is as expected: eyes with intravitreal injection of 1 µl at 5µM NVP-LBG976 showed less choroidal neovascularization than contralateral control eyes.

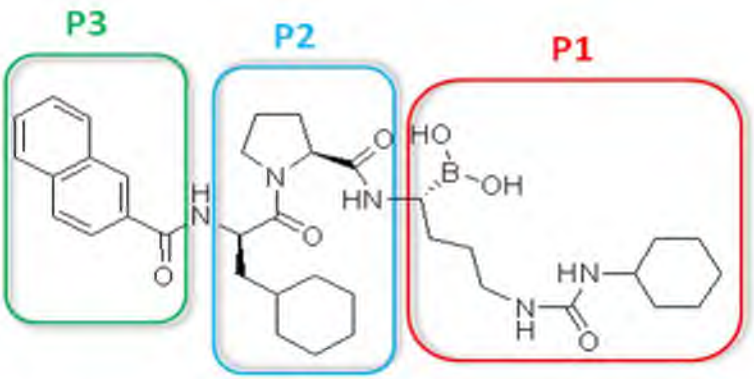
The results clearly showed that small molecule NVP-LBG976 could achieve the in vivo efficacy in multiple animal models of retinal vascular disease. It is a feasible treatment by using HTRA1 small molecule inhibitors.

For aim 2: Structure-activity-relationship study of HTRA1 inhibitor NVP-LBG976 for novel small molecules with better in vitro and in vivo efficacy.

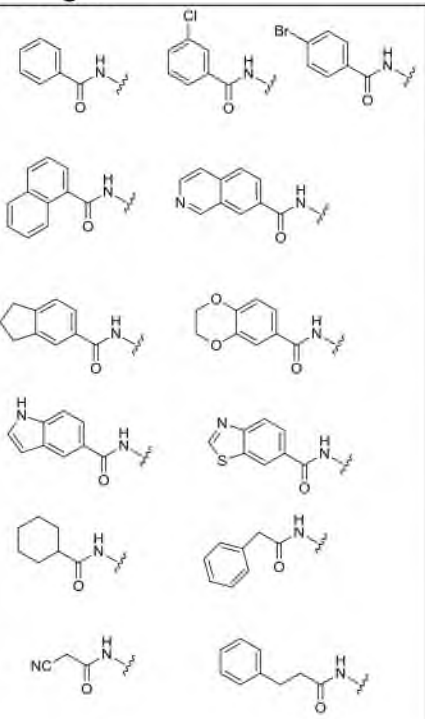
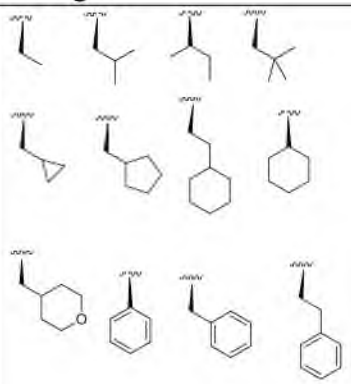
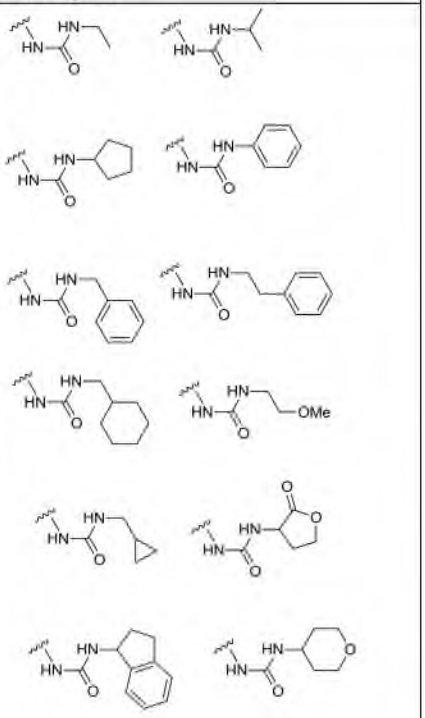
In the aim 1 studies, we got the positive result in proving concept of HTRA1 inhibitor. LBG976 was a good starting point for us to use the medicinal chemistry strategies to improve its potency and efficacy, and the established synthesis protocols allowed us to synthesize those new molecules efficiently.

Subaim 2a: Design and synthesis the focus library based on the structure of NVP-LBG976.

We tried to modify NVP-LBG976 in P1, P2 and P3 regions. When modifying one of the regions, the substitutions in the other two regions were kept as same.



The following substitutions were made for each region:

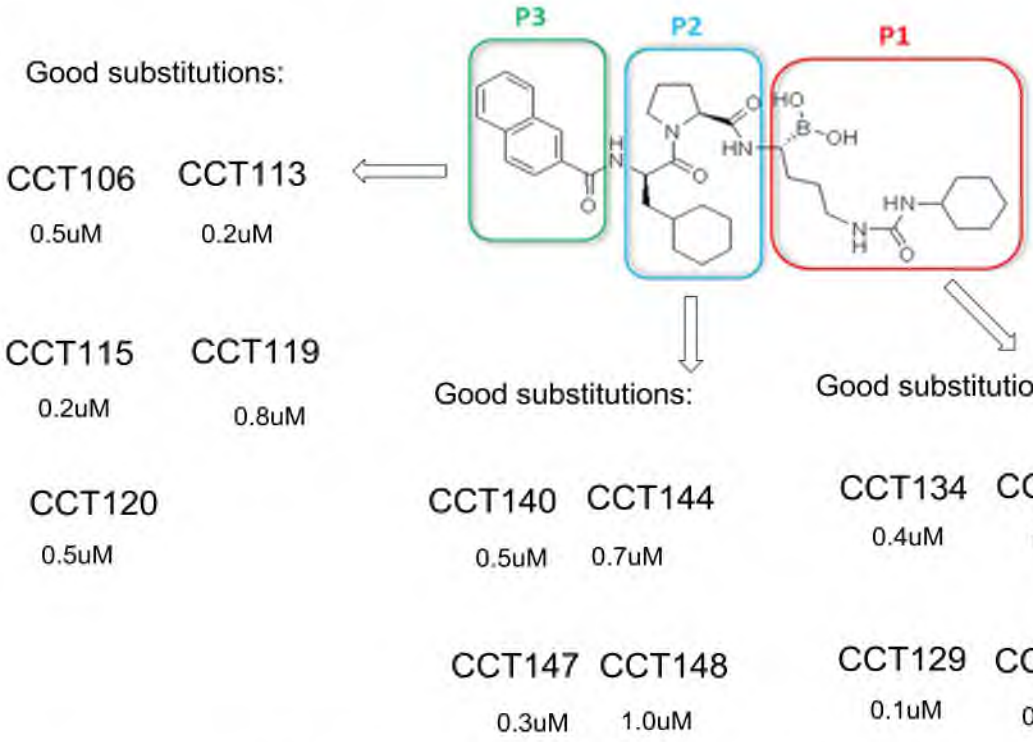
P3 region	P2 region	P1 region
		

By combination of 3 regions, we totally made 65 compounds for this focused library.

Subaim 2b: Screen designed focus library with invitro HTRA1 proteolytic activity blocking assay;

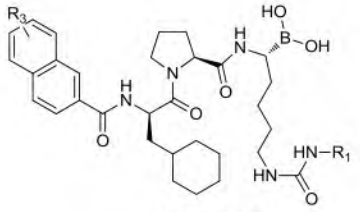
The protease activity blocking assay was used to screen the focus chemical library. 5μg HtrA1 was pre-incubated for 20 min at room temperature with various concentrations of the small molecules (10 points dilution, 40μM, 13.3μM, 4.4μM, 1.5μM, 0.5μM, 0.16μM, 0.05μM, 0.02μM, 0.006μM, 0.002μM). 60 μg of resorufin-labeled casein (Roche) universal protease substrate was added to each sample. Samples were incubated at 37°C overnight in the dark. The reaction was

stopped by trichloroacetic acid precipitation. After centrifugation, the supernatant (400 µl) was mixed with 600 µl of 0.5 M Tris•HCl, pH 9.5, and the absorbance was determined at 574 nm. NVP-LBG976 and DMSO solution were used as controls. The concentration of small molecule that can block 50% of HTRA1 activity (ID50) were calculated. The good substitutions in each region were summarized as below:

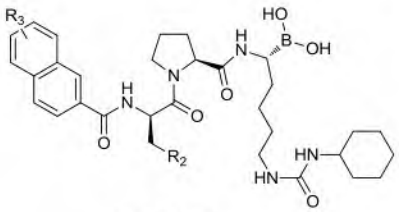


Subaim 2c: Viladate the improved efficacy of the new designed small molecules in animal models of retinal vascular disease.

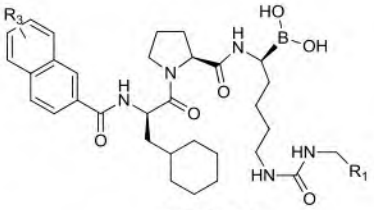
The following top 5 molecules (CCT113, CCT129, CCT152, CCT155, CCT157) were further confirmed in biochemical assays and 3 of them (CCT152, CCT155 and CCT157) were tested in OIR and CNV models. All three compounds showed better efficacy than NVP-LBG976 in OIR model, but only CCT157 worked better in CNV model. The best compound CCT157 showed 3.5 fold improved potency in vitro and 2 fold improved efficacy in vivo than NVP-LBG976.



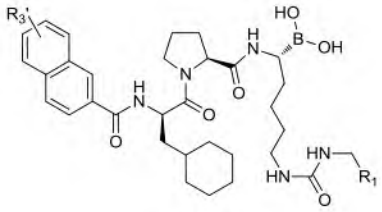
CCT113
0.2uM



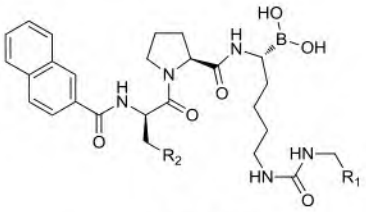
CCT152
0.07uM



CCT129
0.1uM



CCT155
0.04uM



CCT157
0.04uM

Conclusion:

CalCyte team has finished all of the planed studies for this SBIR grant on time. The team validated the efficacy of HTRA1 inhibitor NVP-LBG976 in animal models of retinal vascular disease (OIR and CNV models). The team established the efficient synthesis route to synthesize and modify NVP-LBG976 scaffold. We further did a preliminary structure-activity-relationship study of NVP-LBG976 and totally synthesized 65 analogs and get several comparable or better in vitro active compounds. 3 compounds were tested in animal models of retinal vascular disease (OIR and CNV models) and so far compound CCT157 showed 3.5 fold improved potency in vitro and 2 fold improved efficacy in vivo than NVP-LBG976. Calcyte team achieved the major milestone for Phase I funding, which is the identification of novel small molecules with better efficacy in inhibiting HTRA1 proteolytic activivity and in retinal vascular disease animal models. The results will serve as a springboard for developing a small molecule drug that is effective in reducing pathologic neovascularization of AMD and other retinal vascular diseases in the future.